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CROTOXIN: STRUCTURAL STUDIES, MECHANISM OF ACTION AND CLONING OF ITS GENE

FINAL REPORT

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The purpose of this project is to (1) gain greater insight into crotoxin and crotoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an *in vitro* system for examining presynaptic neurotoxin mechanism of action; (3) clone the crotoxin gene as a first step in creating a non-toxic, but immunoreactive crotoxin analog; and (4) explore other possible non-toxic, crotoxin immunogens as potential vaccines against crotoxin and its homologs.

Acidic and basic subunits of crotoxin were sequenced and their higher-ordered structure examined by circular dichroism, fluorescence and FTIR. Effect of crotoxin subunit cross-linking on its toxicity and phospholipase activity were examined, as well as the interactions of various poly- and

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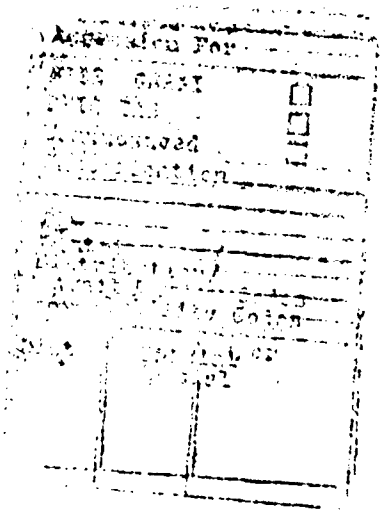
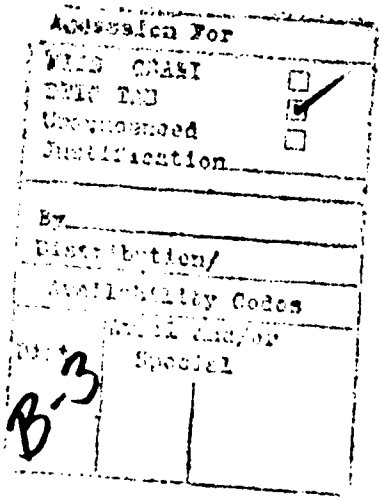
monoclonal antibodies, which were generated and used to examine cro and related toxin structure and function. The first phase of in studies with Dr. Lance Simpson on the effects of crotoxin on iso phrenic nerve-hemidiaphragms has been completed and acetyl choline re from synaptosomes was examined in the absence and presence of neuroto The latter procedure has been plagued with technical problems. Cl studies in conjunction with Dr. Leonard Smith are in progress. Up procedures for isolation and purification of notexin and notechis from N.s. scutatus have been completed and a new toxic, phospholipas been isolated from the venom.





SUMMARY

The purpose of this project was to (1) gain greater insight into crotoxin and crotoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an in vitro system for examining presynaptic neurotoxin mechanism of action; (3) clone the crotoxin gene as a first step in creating a non-toxic, but immunoreactive crotoxin analog; and (4) explore other possible non-toxic, crotoxin immunogens as potential vaccines against crotoxin and its homologs.

Polyclonal antibodies to crotoxin and its subunits from C. d. terrificus, Mojave toxin from C. s. scutulatus, concolor toxin from C. v. concolor, were raised in rabbits. These were used to examine cross-reactivity and cross-neutralization of the above toxins, in addition to searching for other cross-reactive, homologous proteins in other venoms. Four monoclonal antibodies and rabbit polyclonals were raised against the basic subunit of crotoxin and have been further characterized for their cross-reactivity against each other, in the first step of attempting to determine their binding epitopes. One of the monoclonal antibodies is a potent neutralizer of crotoxin's lethality and phospholipase activity. Another, is a non-neutralizer, but is able to inhibit the phospholipase activity by up to 90%. Work is continuing in an attempt to identify the neutralizing MAb epitope and other antigenic regions in the basic subunit of crotoxin recognized by rabbit polyclonal antibodies. A crotoxin homolog is present in the venom of C. v. concolor, C. s. scutulatus (Type A venom), C. vegrandis, and C. d. collilineatus, but none was found in venom from either C. v. lutosis, Lachesis muta muta, or C. atrox/C. atrox-C. s. scutulatus (Type B venom) hybrids. With Lachesis we did demonstrate the presence of a toxic, 60 kd gyroxin-analog, which produced barrel-rolling motions in mice. We have completed the sequence determination of both the basic and acidic subunits of crotoxin. The acidic subunit peptides were difficult, since two of the three peptides were blocked at the amino-terminus by pyroglutamate. Earlier structural studies on crotoxin and related crotalid dimeric, presynaptic neurotoxins indicated that major conformational changes occurred in individual subunits upon formation of the dimeric toxins. Additional studies showed however, that when intact crotoxin was exposed to urea and subsequently re-isolated, their spectra were indistinguishable from unexposed, intact crotoxin. Findings which suggest that the spectral changes observed in isolated subunits were artifacts, resulting from structural changes that occurred during subunit isolation by urea ion-exchange that were not easily reversed under our in vitro conditions. The myotoxin fraction from C. v. concolor was also examined, as a result of earlier indications that there were sequence homologies between these smaller peptides and the basic subunit of crotoxin, as well as the existence of myotoxin isoforms. We did detect multiple isoforms, which all showed qualitatively identical myotoxic activity and behavior in double-immunodiffusion gels against antisera raised against myotoxin *a*. Crotoxin cross-linked by a different cross-linker than used by Hendon and Tu, also proved to be non-toxic. These results

do not rule out however, the possibility that modification of essential residues resulted in the loss of the observed toxicity. What is needed is a cleavable cross-linking reagent that effectively cross-links the two subunits of crotoxin. X-ray crystallography studies with purified Mojave toxin from C. s. scutulatus in collaboration with Dr. Keith Ward of the Naval Research Laboratories are progressing. A manuscript describing the initial structural findings is in preparation. Experiments on a synaptosome system for examining choline and acetylcholine using luminometry are continuing. We have encountered numerous technical problems. The first phase of in vitro studies with Dr. Lance Simpson (Jefferson Medical College), on the effects of crotoxin on the isolated phrenic nerve-hemidiaphragm have been completed and are in press. Our cDNA library from C. d. terrificus was screened for clones of both acidic and basic subunit. We were unable to identify any basic subunit clones and the acidic subunit clones that were partially sequenced, had multiple sequence differences. New cDNA and genomic libraries from C. s. scutulatus are in being prepared and screened in collaboration with Dr. Leonard Smith (USAMRIID). Our preparations of ten purified presynaptic neurotoxins have been completed. We have updated the purification procedure for notexin and notechis II-5 from N. s. scutatus and appear to have found and isolated a new, toxic phospholipase from this venom.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

The investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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STATEMENT OF PROBLEM

The purpose of this project was to (1) gain greater insight into crotoxin and crotoxin homolog structure, in order to provide a better understanding of this class of rattlesnake neurotoxin; (2) develop an in vitro system for examining presynaptic neurotoxin mechanism of action; (3) clone the crotoxin gene as a first step in creating a non-toxic, but immunoreactive crotoxin analog; and (4) explore other possible non-toxic, crotoxin immunogens as potential vaccines against crotoxin and its homologous crotoxin analogs.

BACKGROUND AND APPROACH TO THE PROBLEM

The discovery of crotoxin, a potent, enzymatic neurotoxin from the venom of the South American rattlesnake (Crotalus durissus terrificus) by Slotta and Fraenkel-Conrat (1) marked the beginning of modern-day snake venom research. Crotoxin is a heterodimeric protein, consisting of a moderately toxic basic phospholipase A_2 and a acidic, non-toxic subunit composed of three small proteins (2). The acidic subunit is required for full toxicity, but has no other identified function. Evidence gradually accumulated suggesting similarity between crotoxin, Mojave toxin from Crotalus s. scutulatus, concolor toxin from Crotalus viridis concolor, and vegrandis toxin from Crotalus vegrandis, although disconcerting differences persisted (see ref. 3 and 4 for review). From our early investigations (5), we were reasonably certain that the above four toxins were similar structurally and functionally. We wanted to extend these studies and prepared rabbit antiserum against crotoxin, concolor toxin, and Mojave toxin, as well as against the acidic and basic subunits of crotoxin. These antisera were used to examine the antigenic relatedness of purified crotalid toxins and subunits by both double immunodiffusion and ELISA. We also determined the relative efficacy of antisera raised against the subunits and intact complex of crotoxin in neutralizing the toxicity of crotoxin and related toxins in mice (6).

In addition to crotoxin and crotoxin-homologs found in crotalids, there are a number of other neurotoxic, phospholipase A_2 enzymes present in elapid and viperid venoms. These are of various forms, ranging from monomers (notexin, ammodytoxins, pseudexin), to dimers (ß-bungarotoxin), to trimers (taipoxin), to pentamers (textilotoxin). While they differ greatly in molecular weight and subunits, there are well-established structural similarities between

these neurotoxins (7). Chains carrying the enzymatic activity have extensive regions of amino acid sequence homologies, suggesting that immunological cross-reactivities between the presynaptic neurotoxins may exist. We carried out a systematic study designed to compare the immunological cross-reactivity of ten different phospholipase A_2s, using purified toxins and rabbit antisera raised to these toxins. ELISAs were used to assess degrees of immunological cross-reactivities.

We also prepared monoclonal antibodies (MAbs) to crotoxin. These have provided us with additional tools to probe crotoxin structure and we were fortunate to generate a neutralizing monoclonal antibody, which may provide for either the development of an anti-idiotype vaccine, or the identity of a neutralizing antigenic site on crotoxin (8,9). We have now partially characterized four different MAbs raised to the basic subunit of crotoxin, with respect to their epitope binding sites on crotoxin. Their effects on crotoxin's phospholipase activity and neurotoxicity have also been probed. Studies with the MAbs are continuing.

We have also examined the venom from the Great Basin rattlesnake (C. v. lutosis), the Uracoan rattlesnake (C. vegrandis), the Brazilian C. d. collilineatus, and the Western diamondback rattlesnake (C. atrox), as well as Western diamondback-Mojave rattlesnake (C. s. scutulatus) hybrids, for crotoxin-like neurotoxins. One publication has resulted from the Uracoan study (Kaiser and Aird, 10); one on the Great Basin rattlesnake (Aird, Seebart, and Kaiser; 11), the Western diamondback results have just been published (Aird, Thirkhill, Seebart, and Kaiser; 12), and the C. d. collilineatus results are in preparation. Only in the two South American rattlesnakes, the Uracoan and C. d. collilineatus did we find crotoxin homologs.

Crotoxin has been the most extensively studied and characterized rattlesnake neurotoxins. When this work was started, the amino acid sequence of its basic subunit was largely determined, with the exception of the Asp/Asn and Glu/Gln residues, which could not be differentiated (13). We completed the sequence of the basic subunit, and have published those results (Aird, Kaiser, Lewis, and Kruggel; 14 and 15). All three acidic subunit chains have now been sequenced and the results have either been published or submitted for publication (2, 16, 17). Repeated attempts to sequence the blocked, amino-terminal end of the B-chain by conventional methods were unsuccessful. In 1987, we initiated a collaboration with Dr. Donald F. Hunt at the University of Virginia, whose laboratory employs tandem mass spectrometry for determining amino acid sequences in proteins. He was recently able to provide us with the amino-terminal sequence of the B-chain. Sequencing of the three peptides present in the acidic subunit, two of which are blocked by pyroglutamate, represents a significant contribution, since others unsuccessfully attempted to sequence the peptides for the past fifteen years.

Both subunits of crotoxin have sequence homology with phospholipases A_2 even though the acidic subunit consists of three separate chains linked by disulfide bonds. Only the basic subunit however, which consists of a single polypeptide chain, actually manifests phospholipase activity.

A very striking conformational change reportedly occurs in crotoxin upon complex formation (18). Marked changes in both fluorescence and circular dichroism (CD) spectra were reported by Hanley (18) to appear when the subunits interact, suggesting a large change in the structure of either one or both subunits. In contrast, when the homologous toxin from C. s. scutulatus venom (Mojave toxin) was examined, no evidence for such a conformational change was noted (Tu et al., 19). Estimates of secondary structure for these proteins conflict with x-ray crystallographic data from non-toxic, homodimeric phospholipases. In general, phospholipases A_2 appear to have approximately 50% alpha-helical structure (20), while estimates from CD and Raman studies give values of 12-18% and 70% for the C. d. terrificus and C. s. scutulatus basic subunits, respectively. Thus there is an apparent conflict in the literature regarding the existence of both conformational changes upon complex formation and the secondary structure of the subunits.

To resolve these uncertainties, we examined the spectral properties of purified neurotoxins and their subunits from four crotalid taxa, using CD, Fourier Transform infrared (FTIR), and fluorescence spectroscopy. We also found evidence that could suggest conformational changes in the isolated subunits upon dissociation in all cases examined and evidence that the secondary structure of these toxins differed from those predicted for non-toxic venom and pancreatic phospholipases A_2. Further studies of crotoxin, however, suggested that the apparent conformation changes arose primarily from the presence of urea during the subunit isolation process (21).

We have also examined the myotoxin fraction from C. v. concolor. Goncalves (22) reported the presence of a highly basic polypeptide from the venom of C. d. terrificus, which he named crotamine. Since that time, crotamine-like proteins have been reported in the venoms of a number of different crotalids. Although the exact biological mode of action of these myotoxins is not known, it is clear that their first microscopically observable effect is on muscle cells, causing vacuolation (Ownby et al., 23). The purpose of the work by us was to determine whether the minor structural differences between isotoxins isolated from one batch of venom resulted in any biological and immunological differences. This has implications to our work on crotoxin, because of the crotoxin and crotoxin-homolog isoforms recently identified by us and others (11, 24). In addition, earlier sequence comparisons suggested that there were some sequence homologies between these smaller peptides and the basic subunit of crotoxin.

Previous work by Hendon and Tu (25) was designed to examine whether dissociation was essential for neurotoxicity. Dimethyl suberimidate (DMS), was used to covalently bind the two subunits. Their data suggested that they introduced an average of three crosslinks per complex. At least one of these must have been between subunits because recovered complex could not be dissociated in 6M urea. Sequence analyses indicate the presence of 10 lysine residues (the most likely reactant) in the basic subunit and one each in the A-chain and B-chain of the acidic subunit (2). The DMS-crotoxin had comparable levels of phospholipase A_2 activity to that of

unmodified crotoxin (21 μmol/min-mg). LD_{50} values in mice increased from 0.06 μg/g to >1.5 μg/g. Retention of phospholipase activity and loss of neurotoxicity in the cross-linked complex was interpreted to reflect "interference between the cross-linked complex and the target site." Recent results from three different groups indicate that loss of neurotoxicity may have resulted from modification of the ε-amino group of lysine and not necessarily cross-linking. Using chemical derivatization techniques, Rosenberg's group (26) observed that lysine or arginine group modification in basic phospholipase A_2 enzymes, frequently results in greater loss of pharmacological than of enzymatic activity. They note that modification of these basic amino acids may alter the protein's stability, distribution, or tissue binding ability. Jeng and Fraenkel-Conrat (27) also observed inactivation of neurotoxicity in crotoxin upon acetylaton of amino groups and suggested that the inactivation was due to a "discrete change in the conformation of the molecule induced by the loss of positive charges." In work with biotinylated-crotoxin, we observed that biotinylation using N-hydroxysuccinimido-biotin resulted in a greater than 15-fold decrease in the LD_{50}. Since both the activated biotin and DMS react primarily with ε-amino groups of lysine, the results suggested that loss of toxicity observed by Hendon and Tu (25), might have resulted from loss of essential amino side chains and be unrelated to cross-linking per se.

Hendon and Tu also used DEAE-cellulose column chromatography to separate cross-linked and unreacted toxin components in phosphate-buffered urea. Their neurotoxicity loss may have resulted, in part, from carbamylations during chromatography resulting from spontaneous breakdown of urea generating cyanate which reacts with amino groups. Their unreacted controls were not cycled over urea columns. We repeated Hendon and Tu's cross-linking experiments with the appropriate controls, and screened a number of different cross-linkers. The most effective was found to be a water soluble carbodiimide, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), which activates carboxy side chains, thereby enabling strategically located ε-amino groups of lysine to form internal covalent links in the absence of any "linker" (28). Water soluble carbodiimides can also react with hydroxy groups on tyrosines, but these are presumably removed by hydroxylamine treatment (29). Cross-linked crotoxin was isolated by gel filtration in the presence of urea and a number of its properties examined.

Samples of purified Mojave toxin have been provided to the laboratory of Dr. Keith Ward (Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, D. C.) for crystallization studies. His laboratory has been successful in obtaining crystals suitable for x-ray diffraction studies (Norden et al., 30). Detailed x-ray structural analysis should provide valuable insights into rattlesnake presynaptic neurotoxin structure.

Our progress involving the cloning of the crotoxin gene has been fraught with difficulties. We have had several false starts with cDNA clones from C. d. terrificus. We amplified cDNA using the polymerase chain reaction, inserted the product into Bluescript II KS plasmids and

transformed E. coli. Partial sequencing of several clones indicated that they contained acidic subunit cDNA. In addition, all sequences had base changes that may have been introduced durin the polymerase chain reaction amplification. We were also unsuccessful in identifying any clones that contained basic subunit cDNA. Work is continuing with new cDNA and genomic libraries from C. s. scutulatus, in collaboration with Dr. Leonard Smith at USAMRIID.

We thought that at this stage of our research, that we would have available an in vitro system of synaptosomes and/or tissue culture cells with well defined responses to crotalid neurotoxins. We have conducted experiments using guinea pig brain synaptosomes and ^{3}H-choline uptake and release measurements to monitor responses to crotalid neurotoxins. Existi assays and procedures have proven to be more extensive and involved than originally anticipated. New methodology, involving luminometry is being examined and attempts are beir made to use this method in conjunction with synaptosomes as a system for studying presynapti neurotoxin action in vitro.

We have also initiated a collaboration with Dr. Lance Simpson (Jefferson Medical College) who has examined crotoxin for its neuromuscular blocking properties on the isolated phrenic nerve-hemidiaphragm preparation from the mouse. We have probed the action of crotoxin, using procedures that Simpson has employed successfully with clostridial neurotoxins. In wo to date, we have added the intact toxin or the individual subunits to the isolated phrenic nerve hemidiaphragm preparation. This was done in the presence or absence of drugs or procedures known to alter the progression of clostridial neurotoxin-induced paralysis. Results have provided additional insights into the mechanism of action of phospholipase A_2 neurotoxins (see ref. 31).

We have completed our isolation and purification of 100 mg of each of ten different presynaptic neurotoxins. These have been provided to Dr. John Middlebrook at USAMRIID. Toxins isolated included pseudexin, notexin, taipoxin, textilotoxin, ß-bungarotoxin, concolor toxin, ammodytoxin, caudoxin, Mojave toxin and vegrandis toxin.

RESULTS

I. POLYCLONAL ANTIBODIES.

A) Antisera to crotoxin, its subunits, and related toxins. Antisera were raise against intact crotoxin (C. d. terrificus), Mojave toxin (C. s. scutulatus) and concolor toxin (C. concolor), as well as the subunits of crotoxin. Double immunodiffusion and enzyme-linked immunosorbent assays (ELISA) demonstrated antigenic similarity between these three purifie toxins and their subunits. Additionally, when crotoxin antisera were pre-incubated with each of the three toxins before injection, the lethal activity of all were neutralized equally well.

Antiserum was considerably more effective in neutralizing crotoxin in vivo when the toxin was injected i.m. than when injected i. v. Antisera against both intact crotoxin and its basic subunit were an order of magnitude more effective than crotoxin acidic subunit antiserum in crotoxin neutralization. Purified phospholipase A_2 from C. adamanteus and C. atrox showed weak cross-reactivity with antisera raised against intact crotoxin and its subunits in the ELISA. Our results suggest that crotalid neurotoxins can be detected and neutralized by polyclonal antibodies raised against any intact toxin or basic subunit in this group of homologous toxins. See the manuscript by Kaiser et al. (6).

B) Antisera raised to other presynaptic, phospholipase A_2 neurotoxins. Polyclonal rabbit antisera were raised against ten snake phospholipase A_2 neurotoxins and one snake phospholipase A_2 cytotoxin. Immunological cross-reactivities between these toxins, two other snake phospholipase A_2 enzymes and pancreatic phospholipase A_2 were studied using ELISA technology. All snake phospholipase A_2 neurotoxins fell into two main antigenic classes. One antigenic class was composed of all the elapid toxins tested (textilotoxin, taipoxin, notexin, pseudexin and ß-bungarotoxin), the cytotoxic phospholipase A_2 from Naja naja atra and pancreatic phospholipase A_2. Beta-bungarotoxin seemed to be in an immunological subclass of its own compared to the rest of the elapid toxins. The second antigenic class was comprised of crotalid and viperid phospholipase A_2 neurotoxins (crotoxin, concolor toxin, Mojave toxin, vegrandis toxin, ammodytoxin and caudoxin). Our data indicated that the viperid toxins, caudoxin and ammodytoxin, were immunological subclass apart from the crotalid toxins. In all cases, the homologous antisera were effective in protecting mice from the lethal effects of the toxins. In certain instances heterologous antisera could also protect. These data suggest that the snake phospholipase A_2 neurotoxins have common epitopes and, in some instances, common neutralizing epitopes. Additional details of this work may be found in reference 32 by Middlebrook and Kaiser.

II. MONOCLONAL ANTIBODIES. A manuscript describing the preparation of a crotoxin neutralizing monoclonal antibody and three non-neutralizing monoclonal antibodies has been published (Kaiser and Middlebrook, 8). These four different monoclonal antibodies (MAbs) all typed as IgG_1 subclass and were raised against the basic subunit of crotoxin. The neutralizing antibody could neutralize ≈1.6 moles of purified intact, crotoxin per mole of antibody and enhanced the neutralizing ability of commercial polyvalent crotalid antivenom against the lethality of crude C. d. terrificus venom four-fold. Paradoxically, this monoclonal antibody by itself was ineffective against the lethality of crude C. d. terrificus venom. Using an enzyme-linked immunosorbent assay (ELISA), we tested various proteins for competitive inhibition of

binding of biotinylated-crotoxin to plates coated with the four individual MAbs. Concolor toxin, vegrandis toxin, intact crotoxin, Mojave toxin, and the basic subunit of crotoxin showed increasing effectiveness as displacers of crotoxin from the neutralizing MAb. None of the MAbs reacted with purified phospholipase A_2 enzymes from Crotalus atrox or Crotalus adamanteus, nor any of the components present in the crude venoms from four different elapids known to contain presynaptically acting neurotoxins, which show some sequence identity to crotoxin.

As a first step in our epitope analysis for the four MAbs, we examined each in two different types of ELISAs. One involved labeling each MAb with biotin and determining how each individual biotin-labeled MAb competed for the same binding site on its antigen with one of the other MAbs. Alternatively, one of the MAb can be immobilized and a second MAb allowed to first incubate with biotinylated-antigen which was then reacted with immobilized MAb.

Results for the epitope analysis of biotinylated MAb 1, is shown in Fig. 1. In regard to only MAbs, the homologous, non-biotinylated MAb is the most effective competitor in each assay, lending credibility to the assay. Each MAb also appears to have a unique specificity. A summary and semiquantitation of the data are presented in Table 1A, where the interaction between the four MAbs are denoted by + signs. The greater the number of +, the greater the interaction observed. Included in Table 1A are rabbit polyclonal antibodies (P) raised against the basic subunit of crotoxin. In all cases the polyclonal antibodies were a more potent inhibitor of biotinylated-MAb than the unmodified, homologous MAb. Polyclonal antibodies corresponding to the epitope recognized by the MAb are probably present in the Ab mixture, plus additional Ab that recognize adjacent and/or overlapping epitopes. Some MAb could therefore be inhibited by steric inhibition of bound polyclonal antibodies--in certain instances at great distance from the antigenic site. Rabbit polyclonal Abs also appear to recognize additional epitopes. In one experiment rabbit polyclonal antibodies raised to the basic subunit of crotoxin was biotinylated and an inhibition experiment performed with a mixture of all four MAbs. Figure 2 shows that the inhibition was limited to about 60%, indicating that rabbit polyclonal antibody recognized additional epitopes on the basic subunit antigen. Presumably then, only a fraction of all possible epitopes are represented by our four MAbs, assuming the original mouse antisera was equivalent to the rabbit antisera.

In a complementary assay, wells are coated with a MAb and biotinylated-crotoxin is mixed in a separate tube containing different dilutions of the test MAb. After overnight incubation, the biotinylated-crotoxin-MAb mixture is added to the wells. If the antigenic site is still available, then significant binding of the biotinylated-crotoxin should occur and subsequently be detected by the avidin-peroxidase conjugate. Epitope analysis using biotinylated-crotoxin and

MAb 1 coated plates is illustrated in Fig. 3 and summarized in a semiquatitative manner in Table 1B. There is excellent correlation between these results and those of the earlier described complementary experiment. This suggests that the biotinylation of either crotoxin or antibody did not significantly affect its binding. Nor in the solid-phase assays does there appear to be conformational changes in either the crotoxin or antibody that affects binding. In these assays, the role of crotoxin and antibody have been reversed which permits the affects of derivatization and binding to be examined. Polyclonal antibodies were again potent competitors for each of the MAbs.

We have also now completed western blots of crotoxin and related proteins from reducing and non-reducing SDS-PAGE gels, using rabbit polyclonal antibodies raised to intact crotoxin and the four MAbs as primary antibodies. Polyclonal antibodies were purified on a crotoxin-Sepharose affinity column and the four MAbs on protein-A columns. Blotting methods were essentially as described (33) with either goat-antirabbit or goat-antimouse conjugated with peroxidase serving as secondary antibodies.

We observed strong signals from the basic subunit of crotoxin with all Abs, whether applied as crude venom, intact crotoxin, or purified subunit--in the absence of reducing agent. When DTT was included in the protein solubilizing solution, strong basic subunit bands were observed with the polyclonal Ab and MAb 11. Progressively weaker signals of the basic subunit were observed with reduced samples with MAbs 2, 1, and 5 respectively.

III. EFFECT OF MONOCLONAL ANTIBODIES ON PHOSPHOLIPASE ACTIVITY. Phospholipase activity is associated with all presynaptic neurotoxins. It was therefore of interest to examine the effects that the neutralizing and non-neutralizing MAbs would have on the phospholipase activity of intact crotoxin, its basic subunit, and a related, non-toxic phospholipase from C. atrox.

Preincubation of MAb 1 with crotoxin at a molar ratio of 1:1, resulted in a potent inhibition of crotoxin's phospholipase activity as shown in Fig. 4. At lower antibody to crotoxin ratios, the inhibition was relaxed somewhat, although even at a 0.125:1 ratio the rate of phospholipid hydrolysis was inhibited by about 25%. By increasing antibody to crotoxin ratios from 1:1 to 4:1 the inhibition was slightly enhanced, although in all cases it was generally greater than 90%. Even in experiments where MAb 1 was added to an ongoing reaction containing crotoxin, phospholipid, and 1% Triton-X100, there was an immediate inhibition of phospholipase activity (Fig. 5). This indicates that the enzyme is available or becomes available for reaction with the antibody in the presence of substrate.

Crotalus atrox phospholipase A_2 at an antibody to phospholipase molar ratio of 4:1 showed no evidence of inhibition, as may be seen in Fig. 6. This is consistent with the lack of

competition shown by this protein for the binding of biotinylated intact crotoxin to MAb 1 and also in ELISAs using immobilized antigens.

Monoclonal antibody 2, while not a neutralizing antibody, showed inhibition of crotoxin's lipase activity similar to MAb 1 (Fig. 7A). Monoclonal antibodies 5 (Fig. 7B) and 11 (not shown), both displayed partial inhibition of crotoxin's lipase activity but substantially less than lines 1 and 2.

We were surprised to find that unlike intact crotoxin, phospholipase activity associated with the isolated basic subunit was not inhibited well under the same conditions that led to potent inhibition of intact crotoxin phospholipase activity (preincubation at a 1:1 molar ratio) with MAb 1. Fig. 8 illustrates this difference in inhibitory susceptibility, and should be compared with Fig. 4. In different experiments, we observed from 30-50% inhibition of basic subunit phospholipase activity, when preincubated with MAb 1 at a 1:1 molar ratios. This suggested that the presence of the acidic subunit was necessary for potent inhibition by MAb 1. To examine the role of the acidic subunit further, basic subunit was mixed with MAb 1 and preincubated 15' in the absence and presence of a four-fold excess of acidic subunit relative to the basic subunit. The increased phospholipase inhibition in the presence of the acidic subunit is dramatic (Fig. 9). In Fig. 5 we observed that addition of MAb 1 to an ongoing reaction of intact crotoxin hydrolyzing phospholipid substrate led to an immediate decrease in its hydrolytic activity. We observed a similar response when a four-fold excess of acidic subunit over basic subunit was added to an ongoing phospholipase assay (Fig. 10). There was an abrupt inhibition of phospholipase activity upon acidic subunit addition.

IV. MOUSE ANTI-IDIOTYPE. Nine anti-idiotype ascites fluids raised against the crotoxin neutralizing monoclonal antibody (line 1), were received from Dr. John L. Middlebrook at USAMRIID. These were stored at -80^{o}C until used. Samples were thawed, spun in a microfuge, and supernatants aspirated. Total protein assays (BioRad) on the supernatants and gave values ranging from 26.6 to 43.3 mg/ml. Supernatants were then run on SDS-PAGE on 7.5% gels for 6 hours at 20 mA. Results are shown in Fig. 11. About 20 µg of protein was run per slot. Phospholipase assays (Aird and Kaiser, 5) were run directly on each ascites fluid supernatant, with no evidence of any activity. An attempt to purify each monoclonal antibody from its ascites fluid was then carried out by a combination of ammonium sulfate precipitation and Protein A affinity chromatography (8). Yields were low in all cases, as expected, since the gels on the crude ascites did not show significant quantities of antibodies in the ascites fluids (see Fig. 11). Nevertheless, we were able to isolate small amounts of anti-idiotype monoclonal

antibodies 2, 4, 6, and 9. These were examined by SDS-PAGE (Fig. 12), and assayed for phospholipase activity. No phospholipase activity was found in any of the four purified anti-idiotype monoclonal antibodies examined.

V. SEARCH OF DIFFERENT VENOMS FOR CROTOXIN HOMOLOGS.

(A) CROTALUS VEGRANDIS. A major protein toxin from the venom of Crotalus vegrandis was examined by gel filtration, anion-exchange chromatography, and SDS polyacrylamide gel electrophoresis. The toxin was separated into several isoforms by ion-exchange chromatography and spontaneously dissociated into free acidic and basic subunits, mimicking the behavior of crotoxin. Rabbit antisera raised against crotoxin reacted strongly in enzyme-linked immunosorbent assays with the intact C. vegrandis toxin isoforms and their basic subunits, and formed precipitin lines of identity with intact crotoxin in double immunodiffusion gels. These results indicate that vegrandis toxin is strongly homologous with crotoxin from the venom of Crotalus durissus terrificus. Experimental details may be found in the published paper (10).

(B) CROTALUS DURISSUS COLLILINEATUS. In their paper on isoforms of crotoxin, Faure and Bon (24) reported that crotoxin-like material "was a minor component or even absent in venoms" from C. d. collilineatus. Contrary to this report, we found significant amounts of crotoxin-like material in three different lots of C. d. collilineatus. In fact, all samples showed that the crotoxin-like material represented more than 40 % of the total venom protein. In one lot it approached 80 %. The crotoxin-like material was isolated and compared with crotoxin using a variety of techniques, including gel filtration on Sephacryl S-200SF, SDS-PAGE, isoelectric focusing, phospholipase activity, lethality, immunological reactivity and anion-exchange chromatography. All results indicated that the principal toxic component in C. d. collilineatus venom was a homolog of crotoxin. A manuscript describing this work is in preparation.

(C) CROTALUS VIRIDIS LUTOSUS. Crude venom from Crotalus viridis lutosus was fractionated over the gel filtration medium Sephacryl S-200. Each fraction was examined by SDS-PAGE and selected fractions by reverse-phase liquid chromatography. Based on these profiles, enzymatic assays, toxicity assays in mice, and immunoassays, we found no evidence for the presence of a crotoxin homolog in the venom (11). This is in contrast with the conspecific taxon, Crotalus viridis concolor (5), held by Klauber to be a stunted offshoot of C. v. lutosus. Experimental details for work on C. vegrandis and C. v. lutosus may be seen in ref. 10 and 11.

(D) CROTALUS ATROX AND CROTALUS ATROX-CROTALUS SCUTULATUS

SCUTULATUS HYBRIDS. This work was recently published in the Journal Of Herpetology (12). Venom composition and morphology of a male C. atrox, a female C. s. scutulatus, their F1 hybrid offspring, and a half sibling C. s. scutulatus were examined. F1 hybrids showed morphological and venom characteristics of both parental species. F2 hybrids showed morphological characteristics of both C. atrox and C. scutulatus. C. s. scutulatus venom (Type B) was shown to be qualitatively similar to that of C. atrox and qualitatively very different from C. s. scutulatus (Type A) based on gel filtration and ion-exchange chromatography, ELISA and PAGE. Guidelines were provided regarding the detection of presynaptic neurotoxins in crotalid venoms to prevent their being confused with non-toxic phospholipases A_2. Several hypotheses were offered to explain the origin of the Type B venom population within C. s. scutulatus. This work has practical importance from the standpoint of venom neutralization using crotalid antisera.

(E) LACHESIS MUTA MUTA. Clinical observations of possible neurotoxic activity in bushmaster (Lachesis muta muta) envenomations, coupled with the accepted ancestral relationship of Lachesis to other crotalids, suggested that Lachesis venom might contain a crotoxin-like molecule. Crude venom and gel-filtration fractions showed modest reactivity in ELISAs using rabbit polyclonal antibodies raised against the basic subunit of crotoxin, but no reaction was detected with a murine monoclonal antibody raised against the same antigen. Phospholipase assays, LD_{50} determinations, and SDS-polyacrylamide gel electrophoresis indicated the presence of non-toxic phospholipases, but no crotoxin homologs. A higher molecular weight, toxic protein (60,000) with an LD_{50} of 0.07µg/g in mice was isolated and purified, which induced gyroxin-like, rapid rolling motions in mice. Its amino-terminal sequence shows considerable amino acid sequence identity with gyroxin from the venom of C. d. terrificus and other serine proteases. A manuscript detailing this work has been published in Toxicon (34).

VI. SEQUENCING OF CROTOXIN.

(A) BASIC SUBUNIT. The complete sequence of the basic subunit of crotoxin was obtained by a combination of direct sequencing from the amino terminus of the pro[illegible] and by sequencing cleavage fragments obtained after digestion with the specific endoproteases Arg-C and Lys-C, or after incubation with cyanogen bromide.

Arg-C and Lys-C both showed variable specificity for their respective target residues and for one unanticipated site. Arg-C consistently cleaved the bond between Tyr-24 and Gly-25 with high efficiency. It hydrolyzed the Arg-Pro bond between positions 35 and 36 poorly, as would be expected, and it repeatedly showed no activity against Arg-42, which is flanked on the carboxyl side by two Cys residues. Arg-C

cleaved with high yields at Arg-14, Arg-37, Arg-65, Arg-90, Arg-97, and Arg-98. Lys-C was unable to cleave the Lys-Trp bond at positions 60-61 and hydrolysis was not particularly efficient at Lys-77. This suggests that the tryptophanyl side group may inhibit the binding of Lys-C. The latter protease was highly effective against Lys-15, Lys-37, Lys-56, Lys-69, and Lys-104.

All of the Glu/Gln and Asp/Asn residues in the Fraenkel-Conrat et al. (13) sequence have been resolved and several changes have been made in that sequence. Most significantly, Tyr-61 of the Fraenkel-Conrat sequence could not be found in our protein. Its absence is consistent with the sequences of all known snake venom phospholipases A_2. Position 33 varies with the source of the venom. Venom of C. d. terrificus obtained from the Butantan Institute possessed Gln at position 33, consistent with the Glx reported by Fraenkel-Conrat. However, venom obtained from the Miami Serperntarium yielded Arg at that position with only a trace of Gln. Additional modifications of the Fraenkel-Conrat sequence are as follows: Glx-12=Glu, Asx-16=Asn, Asx-38=Asp, Asx-41=Asp, Asx-48=Asp, Asx-58=Asn, Asx-63=Asp, Glx-83=Glu, Glx-84=Glu, Glx-85=Gln, Glx-88 =Glu, Asx-90=Asp, Glx-95=Glu, Asx-100=Ser, and Asx-106=Tyr. See ref. 14 for experimental details.

(C) **ACIDIC SUBUNIT.** The acidic subunit of crotoxin consists of three chains, interconnected by disulfide bonds. After reduction and carboxyamidomethylation, the chains were readily separable by reverse-phase HPLC, and located by a post-column fluorescamine detection system that detects free primary amines. C-chain, which lacks Lys, was undetectable if deblocking of its amino-terminal end with pyroglutamate aminopeptidase was omitted. It contains 14 residues, as anticipated from composition data (35).

Comparisons with 3251 sequences showed strong homologies to portions of snake venom phospholipases A_2. Sequence homologies between the acidic subunit and nontoxic crotalid phospholipases yield Z scores of 16.4, 12.0, and 7.5 for the A, B, and C chains, respectively (2).

The A chain has retained both the Tyr-Gly-Cys-Tyr-Cys-Gly-(Trp)-Gly-Gly segment (positions 24-32), common to all known snake venom phospholipases, and the Asp-Arg-Cys-Cys-Phe-(Val)-His-Asp-Cys-Cys-Tyr-(Gly) segment (positions 41-52), which is invariant among crotalid and viperid phospholipases. B chain homologies with phospholipase segments are also very high in positions 71-104. Locations of four to five cysteine residues have been maintained, two of which occur in

the highly conserved segment Cys-Asp-Lys-Ala-Ala-Ala-Ile-Cys-Phe-Arg (positions 90-99). The 14-residue C chain is homologous with the carboxy terminus of viperid and crotalid phospholipases, retaining both cysteines at positions 118 and 125. It also shows high homology with a segment (residues 48-61) of mammalian neurophysins and their precursors. Experimental details relevant to sequencing of the acidic subunit may be found in ref. 2.

A partial sequence for the B-chain in the acidic subunit was reported in the above reference. The N-terminus was blocked by pyroglutamate, although the residue was refractory to the enzyme pyroglutamate aminopeptidase. The sequence was determined by a combination of Edman degradation and tandem mass spectrometry, in collaboration with Dr. Don Hunt at the University of Virginia. The B-chain contained 35 amino acids and showed 91 % amino acid identity with the corresponding segment from Mojave toxin, a homologous neurotoxin from C. s. scutulatus, and is shown below.

pEEDGEIVCGEDDPCGTQICG/ECDKAAAICFRNSMDT

The sequence of the last 24 residues of the B-chain was consistent with that previously published (2), except at position 20, where Edman degradation gave glycine and mass spectrometry gave glutamic acid. A manuscript describing the details of the sequence determination has been submitted for publication (17).

VII. SPECTRAL MEASUREMENTS. A preliminary report on the spectroscopic studies of crotoxin and its homologs has been made (36). Over the past year additional studies have been carried out, which have led to substantially different conclusions than were reached earlier. Briefly, structures of four related neurotoxins and their purified subunits from the venoms of C. d. terrificus, C. vegrandis, C. s. scutulatus, and C. viridis concolor were examined by circular dichroism (CD), deconvolution Fourier-transform infrared (FTIR), and fluorescence spectroscopy. CD spectra suggest that in general, the isolated subunits were decreased slightly in α-helix, while increased in ß-sheet structure, relative to intact toxins. These results were consistent with FTIR results. Fluorescence quenching (50-80%) was also observed in three of the four intact toxins as compared to spectra predicted by summation of free acidic and basic subunit spectra. It was tempting to conclude from these results that major conformational changes occur in individual subunits upon formation of the dimeric toxins. Intact crotoxin, however, when exposed to urea and then reisolated, yields spectra (CD, FTIR, and fluorescence) that are virtually identical to control intact crotoxin. These findings suggest that the enhanced fluorescence exhibited by the isolated subunits, as well as the secondary structural changes in α-helix and ß-sheet, are artifacts resulting from structural changes that occur during subunit

isolation by urea ion-exchange chromatography, which are not reversible under our in vitro conditions of subunit assembly. In spite of these structural changes, LD_{50}-values of intact crotoxin reassembled from isolated subunits are unaltered from those of native crotoxin. This work has just been published (21).

VIII. MYOTOXINS. Myotoxins from C. v. concolor were isolated by gel filtration. The crude myotoxin peak was subfractionated into either two or four subfractions by cation exchange FPLC, depending upon the source of the venom. When injected at 2 µg/g, crude concolor myotoxin caused vacuolation of mouse muscle cells typical of myotoxin a from C. v. viridis and crotamine from C. d. terrificus. All four subfractions showed qualitatively identical myotoxic activity. In double immunodiffusion studies, myotoxin a antiserum produced lines of identity when reacted with myotoxin a, crude concolor myotoxin, and the four concolor subfractions. A second batch of material showed two major components when subfractionated by cation exchange FPLC. The more basic of these two components displayed approximately twice the intravenous lethality of the more acidic component. The LD_{50} for the basic component lies between 0.625 µg/g and 0.75 µg/g while that of the acidic component falls between 1.00 µg/g and 1.25 µg/g. For additional details, see reference by Ownby, Aird, and Kaiser (37).

IX. GYROXIN ANALOG. See V (E) section under Lachesis muta muta.

X. CROSS-LINKING OF CROTOXIN SUBUNITS. Four different protein cross-linkers were screened for their reactivity with crotoxin. These include dimethylsuberimidate (DMS), ethylene glycolbis(succinimidylsuccinate) (EGS), disuccinimidyl tartarate (DST), and the water soluble carbodiimide 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC). We concentrated on optimizing the extent of crotoxin subunit crosslinking with each of these reagents, and have had reasonable success with all but EGS, which gave only low levels of subunit crosslinking under a variety of conditions. Subsequent studies suggested that EDC reacted well with crotoxin, and was therefore selected as the cross-linker of choice. A manuscript entitled "Effects of subunit cross-linking on the properties of crotoxin" has been accepted for publication (38). In this paper crotoxin was covalently cross-linked using EDC. Gel filtration of the reaction mixture in 8M urea gave rise to several peaks not observed with native crotoxin and permitted separation of cross-linked crotoxin. This material had the expected amino-terminal amino acids, amino acid composition, behavior on SDS-PAGE and an 80% reduction of reactable lysine residues. It was also non-toxic, had reduced immunological cross-reactivity toward both poly- and monoclonal antibodies raised to the basic subunit of crotoxin and had lost >95% of its phospholipase activity. This work is in keeping with the results of Hendon and Tu (25), which suggested that subunit dissociation was required for

expression of neuro >xicity. It does not, however, rule out the possibility that the loss of toxicity is due to the modification of essential residues rather than subunit cross-linking.

XI. CLONING. We have had several false starts with our cloning work, virtually all of which has been carried out in collaboration with Dr. Smith at USAMRIID. Initially we attempted to clone the crotoxin gene from the Uracoan rattlesnake, Crotalus vegrandis. An adult C. vegrandis snake was milked, four days later sacrificed, and its venom glands dissected and quickly frozen in liquid nitrogen. The glands, weighing about 0.5 g were subsequently extracted for total glandular RNA using the phenol procedure and teflon-glass homogenization. Poly A containing RNA was enriched by passage over an oligo (dT) column and used for cDNA cloning. Enriched poly A containing RNA was annealed to a puc9 plasmid vector following linearization with restriction enzyme Pst I and addition of oligo (dT) tails using terminal transferase. The primary copy from the messenger RNAs was catalyzed by AMV reverse transcriptase. Second strand synthesis was performed using RNAse H to remove the RNA and DNA pol I to copy the first strand. T_4 DNA ligase joined the ends and the constructed molecules were used to transform E. coli. A number of clones were randomly picked, grown up, plasmid DNA extracted, and restricted with a double digest of Hind III and Bam HI to check the size of the inserts. When run on gels, we observed that the inserts were very large and had similar size. We synthesized three oligonucleotide probes to three different areas of the toxin; one to the acidic subunit and two to the basic subunit. These were labeled with ^{32}P and used to probe some of the clones. Six clones grown up on nitrocellulose filters were positive. Growth characteristics and ELISAs run on these different clones suggested that the crotoxin gene had been cloned and that it was being expressed, but not processed by E. coli. Subsequent subcloning and sequencing of pertinent regions of the cloned snake DNA did not reveal the presence of the crotoxin gene. Our most likely problem with this initial experiment was low efficiency of mRNA extraction from the venom gland. Various methods for improvement of this process were subsequently examined in Dr. Smith's laboratory. In our next attempt, five adult Crotalus durissus terrificus snakes were obtained from Herptofauna, Fort Myers, Florida. These animals were milked and sacrificed three days later. Venom glands and liver were surgically removed and quickly frozen in liquid nitrogen. They were shipped on dry ice to to Dr. Smith. Total RNA (375µg) was extracted from 1 gram of gland tissue, which yielded 5µg of poly (A+) RNA. A cDNA library was constructed in a lambda vector using 2µg of the poly (A+) RNA. The library yielded a titer of 3.4×10^6 pfu/ml. Two probes were synthesized (one for acidic and one for the basic subunit of crotoxin) and used to screen 500,000 primary clones. Fifteen acidic subunit positives were picked on the first round of screening. The second round screening of these fifteen yielded 8 strong positives.

Eight were subcloned into a plasmid vector and mapped. The synthetic probe designed for the basic subunit was used to screen 500,000 primary clones. No positive, basic subunit clones resulted from this screening, although the C. d. terrificus library was used by Dr. Catherine Alcaide, working in Dr. Smith's laboratory, for probing and isolating the crotamine cDNA. Eight hundred positives were detected in a first round screening of 400,000 clones. Ten were picked and eight strong positives resulted from the second round screening.

There is about 50 percent sequence identity between C. atrox phospholipase A_2 (122 amino acids) and the basic subunit of crotoxin, although the C. atrox phospholipase A_2 possess no neurotoxicity. It may eventually be possible to combine different gene segments of the basic subunit of crotoxin and the non-toxic C. atrox phospholipase A_2 to form a "hybrid" phospholipase and identify toxic domains in the presynaptic neurotoxin. With this in mind and also to gain some experience in manipulating Crotalus DNA, we initiated studies to clone the phospholipase A_2 gene from C. atrox.

To date, we have successfully (1) isolated C. atrox genomic DNA, (2) probed total genomic DNA in a dot-blot assay, and (3) carried out southern blots of agarose gels of genomic DNA restriction enzyme digests. Our probe consisted of a redundant synthetic 17-mer (53-76% GC; 64 degeneracy) complementary to the C. atrox phospholipase antisense strand, corresponding to an internal amino sequence from Tyr-27 to Gly-32. This sequence is identical to the corresponding sequence in the basic subunit of crotoxin. Complete BamHI digests of genomic DNA release a 20kb fragment containing the phospholipase gene. This fragment will be eluted from the gel, ligated and packaged into EMBL3, a lambda replacement vector. Future goals include restriction mapping, sequencing, and finally orientation of the gene in the proper position in an expression vector to attempt initial translation in a bacterial system. Because of the presence of seven disulfides in the phospholipase, it seems unlikely that a fully processed, mature and active phospholipase can be recovered from a bacterial system.

In a third attempt, mRNA isolated from the venom gland of C. d. terrificus, was reverse transcribed. This product was amplified using the polymerase chain reaction and cloned into Bluescript II KS plasmids. Clones with inserts were partially sequenced, using the double-stranded method. Initially we had technical problems with the sequencing as a result of extensive higher ordered structure that was giving us hesitation points in the sequencing ladder. Reaction conditions were adjusted to compensate for these difficulties. We found however, that all clones were acidic subunit clones. About six of these were partially sequenced and they were clearly acidic subunit clones, except that they all showed numerous sequence differences. It was unclear whether these represent different gene products or whether we inadvertently

introduced these differences during the amplification reaction. In view of sequence differences and the absence of any apparent basic subunit clones, we decided to shelve our work with C. d. terrificus and focus on cDNA and genomic libraries from the tissues of the Mojave rattlesnake (C. s. scutulatus).

A cDNA library from C. s. scutulatus has now been prepared by Dr. Smith, and we will prepare a genomic library from liver tissue in our laboratory. Screening of the cDNA library for acidic and basic subunit sequences is in progress. Subcloning, sequencing and expression of clones will be coordinated between Smith's laboratory and ours. We are hopeful that these studies will help resolve some of the questions that have been generated during our work with crotalid neurotoxins.

XII. X-RAY CRYSTALLOGRAPHY OF MOJAVE TOXIN. Mojave toxin is the major presynaptic neurotoxin present in the venom of the Mojave rattlesnake (C. s. scutulatus). We have shown it to be very similar to crotoxin. Single crystals suitable for x-ray structure analysis have been prepared by Dr. Keith Ward (Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, D. C.) from Mojave toxin purified by a combination of gel filtration and ion exchange chromatography. The crystals are orthorhombic, exhibit the symmetry of space group $P2_12_12_1$, and have lattice constraints: a = 38.6, b = 69.9, and c = 77.6 Å. Over 11,000 items of reflection data have been collected to a resolution of 2.1Å. The data have been phased by the molecular replacement method using as a model the structure of dimeric phospholipase A_2 from C. atrox. An atomic model has been refined using Konnert-Hendrickson restrained refinement. The current R = 0.32. The basic phospholipase subunit is related to the nontoxic 'chaperon' subunit by an approximate dyad axis, and is very similar in structure to the C. atrox phospholipase subunit. The relative structural disposition of the three chains of this subunit to that of the phospholipase subunit can now be approximated. This structural information should provide important clues to the mechanism of action of this neurotoxin. A manuscript describing this phase of the work is in preparation.

XIII. IN VITRO ASSAYS TO EXAMINE NEUROTOXIN MECHANISMS. (A) Methods for the estimation of acetylcholine (ACh) and choline (Ch) have been evolving since the early 1900's. As they evolved, the sensitivity of these methods have increased from detecting micromolar to picomolar quantities. Methods have ranged from bioassays, to techniques of colorimetry, spectrophotometry, gas chromatography, HPLC, mass spectroscopy, and radioenzymatic assays. Israel and Lesbats (39) reported a chemiluminescence method for the assay of ACh. This procedure couples ACh to a series of enzymatic reactions with the eventual production of light. A photomultiplier measures the amount of light produced, which is related

to the amount of original ACh present. We spent about six months on this methodology, hoping to be able to monitor the continuous release of ACh from guinea pig brain synaptosomes as reported in the literature. To date, the methodology has proved to be irreproducible in our hands.

None of our studies have revealed the source of irreproducibility. Enzyme ratios and pH influence the chemiluminescence reaction, but do not appear to play a major role in variable results. Neither did changing of the intensity of room lighting, source of either buffer or enzymes, injection method, changing of oxidizing agent concentration, and other assay modifications. Comparison of our borrowed Model 20 Turner Designs luminometer with another instrument of the same type shows no great differences. We have also had LKB provide us with one of their "loaner" luminometers, which apparently had traveled to one to many exhibitions and had numerous electronic and mechanical problems. We are currently re-evaluating the luminometry procedure as it applies to the determination of ACh and Ch.

(B) PHRENIC NERVE SYSTEM WITH LANCE SIMPSON. We have collaborated with Dr. Lance Simpson (Jefferson Medical College) on the affects of crotoxin and its neuromuscular blocking properties on the isolated phrenic nerve-hemidiaphragm preparation from the mouse. The same system has also been used to determine the effectiveness of our neutralizing monoclonal and one non-neutralizing monoclonal antibody, on inhibiting crotoxin's neuromuscular effects. Crotoxin and its two subunits were examined for their neuromuscular blocking activity on the phrenic nerve hemidiaphragm preparation in this system. Two types of experimental approaches were used, the first of which separated the toxin binding step from subsequent events in paralysis and the second of which did not. In both paradigms, the toxin produced concentration-dependent blockade of transmission. However, the results with low concentrations were variable, and in some cases complete neuromuscular blockade did not develop. The isolated acidic and basic subunits possessed little toxicity. In experiments designed to characterize binding, the intact toxin displayed the following properties: i) the apparent half-time for tissue association was about 22 min, ii) binding was not effected by low temperature, the presence or absence of nerve stimulation, and the substitution of strontium for calcium, and iii) when binding was allowed to go to completion, reversibility was negligible. Pretreatment of tissues with the isolated subunits of crotoxin did not enhance or inhibit the binding of the parent molecule. Modification of one histidine residue in the isolated basic subunit, followed by reconstitution with unmodified acidic subunit, generated a molecule with only about 10% of the activity of the native toxin. The modified toxin could not be used to antagonize binding of the native toxin. Both polyclonal and monoclonal antibodies were generated that neutralized the biologic activity of crotoxin. In experiments that separated the binding step

from later events in paralysis, the polyclonal preparation continued to locate and partially neutralize tissue-bound toxin. In experiments that initiated events that follow binding, polyclonal antibodies were progressively less effective with time in neutralizing toxin. The monoclonal preparation did not neutralize toxin after the binding step was complete. This work has been accepted for publication (31).

IVX. PURIFIED TOXINS. During the past year or so, we have been involved in the purification of about 100 mg each of ten different snake venom presynaptic neurotoxins. These correspond to monomeric (pseudexin, notexin, ammodytoxin, caudoxin), dimeric (ß-bungarotoxin, concolor toxin, Mojave toxin, vegrandis toxin), trimeric (taipoxin), and pentameric (textilotoxin) toxins. Availability of these purified samples will enable us to now carry out a number of comparative experiments with crotoxin that were not possible previously, such as those described in references 31 and 32. We expect to use some of this material for studies involving the pharmacology and functionality of this class of snake venom toxins, comparative biochemistry, physical and structural investigations, and immunology.

DISCUSSION

Double immunodiffusion and ELISA studies demonstrated great antigenic similarity between major neurotoxins isolated from C. d. terrificus, C. s. scutulatus and C. v. concolor venoms. Intact toxins and basic subunits of heterodimeric proteins showed clear immunochemical identity on double immunodiffusion plates. Intact toxins also contained determinants that were recognized by antisera to their subunits.

While antisera against intact crotoxin and basic subunit of crotoxin had high neutralization capacities against crotoxin when mixed before injection, such was not the case when i.v. injection of toxin was followed by i.v. neutralizing antiserum. We found that, unless neutralizing antisera was injected within the first minute after crotoxin injection, survival was rare. This indicates that crotoxin is rapidly removed from the circulation and becomes unavailable to neutralizing antibodies. Loss from the circulatory system must be rather selective, however, since the amounts injected (2 LD_{50}) are too small to permit random losses and still observe the high mortality that we do.

To define more completely the immunological relationships among phospholipase A_2 neurotoxins and enzymes, ten purified phospholipase A_2 neurotoxins and one cytotoxin from snake venoms were isolated and used to raise antisera in rabbits. Using ELISAs, we examined the cross-reactivities of the antisera and with the immunizing toxins and three additional toxins or enzymes. The proteins appeared to fall into two antigenic classes. One class was composed of the elapid toxins and pancreatic phospholipase A_2, while the other was made up of the viperid

and crotalid proteins. The first class appears to have two subgroups, ß-bungarotoxin and all the others. Likewise, one could designate two subgroups for the second class as the crotalid toxins or enzymes and the viper toxins. There appeared to be a limited or weak recognition of epitopes on elapid toxins by antisera to the crotalid and viperid toxins, while the converse was not observed. The exception to this rule was ß-bungarotoxin, which was recognized by every antiserum we tested. We found that in general, there was a reciprocity seen with the cross-reactions, in that when antiserum to one toxin produced a moderate to high titer against another, the converse was also true. A practical issue raised by these results is whether the cross-reactivities identified by ELISA may reflect a potential for cross-neutralization. Work is currently in progress in Middlebrook's laboratory exploring this possibility.

Among the MAbs, the homologous MAb was its own best inhibitor; rabbit polyclonal antibodies were at least as effective. Although not particularly strong, the best competitor for MAb 1 was MAb 5, with 2 and 11 showing little competition. MAbs 2 and 11 also showed little competition for 5. MAb 2 and 11 were strong competitors with each other.

These findings suggest that these three MAbs may recognize conformational or non-linear antigenic sites. We should be able to confirm this when we complete our epitope scanning studies using sold-phase peptide synthesis on pin-supports, as developed by Geysen (40). Once the peptides are synthesized, we propose to examine the four MAbs as well as polyvalent Abs for reactivity. It seems likely that this approach will lead to a better understanding of crotoxin-antibody relationships.

The phospholipase active site region from a variety of different snake venom phospholipases A_2, including C. atrox and the basic subunit of crotoxin, shows absolute conservation, including four residues that form a 'catalytic network'. Yet we see no cross-reactivity between any of our monoclonal antibodies and phospholipase A_2 from C. atrox, indicating that the 'catalytic network' residues are not in the epitope recognized by our monoclonal antibodies.

It was surprising to find that both MAbs 1 and 2 showed similar levels of potent inhibition of crotoxin's phospholipase activity (Figs. 4 and 7A); but only MAb 1 exhibited neutralizing activity of toxicity. This inhibition was specific, since under our conditions phospholipase A_2 from C. atrox was unaffected (Fig. 6). Strong et al. (41) showed a maximum of 50% inhibition of phospholipase activity of ß-bungarotoxin with non-neutralizing monoclonal antibodies raised against this toxin, results in keeping with what we found for our monoclonal antibodies 5 and 11.

It is clear that the presence of the acidic subunit enhances the inhibition of phospholipase activity associated with the basic subunit. That this inhibition is enhanced by addition of acidic subunit, suggests that there is association of the acidic subunit and phospholipase-containing basic subunit during phospholipid hydrolysis. What this association is and how it enhances the extent of phospholipase inhibition by MAb 1 is presently unclear.

The gel filtration profile of C. vegrandis venom Sephacryl S-200 suggested the presence of a

crotoxin-like molecule. Double immunodiffusion assays with the three apparent, separable isoforms of vegrandis toxin separated by anion-exchange chromatography demonstrated immunological identity between the isotoxins and with crotoxin. In addition, toxicity determinations confirmed that two of the three peaks had LD_{50}-values similar to that of crotoxin. SDS-PAGE behavior of vegrandis toxin is likewise consistent with other crotalid heterodimeric presynaptic neurotoxins. Based on these results, we conclude that juvenile and adult C. vegrandis venom contains a toxic protein that is structurally and antigenically similar to the major neurotoxins isolated from C. d. terrificus, C. s. scutulatus, and C. v. concolor. It also appears likely that this toxin is the product of a duplicated locus as has been suggested for crotoxin.

Gel filtration and SDS-PAGE indicate that the crude venom of C. d. collilineatus contains a crotoxin-like material. The intact toxin and the subunits have LD_{50} values which are comparable to those of crotoxin. The report by Faure and Bon (24), who found only minimal amounts of crotoxin-like material in venom from C. d. collilineatus, is difficult to reconcile. They may either have been looking at venom from a subspecies that simply did not contain the toxin or perhaps their venom supplier provided them with a miss-labeled sample.

Venom of C. v. lutosus was fractionated over the gel filtration medium Sephacryl S-200. Venom proteins were resolved into five major fractions. Phosphodiesterase and L-amino acid dehydrogenase eluted in the first fraction with estimated molecular weights of 159kD and 133 kD, respectively. Phospholipase activity eluted as a major peak in the second and third fractions with a trailing peak of activity between the third and fourth fractions, with estimated molecular weights of 50.4 kD and 22.2 kD, respectively. These estimates approximate those anticipated for homotetramers and homodimers, and constitute the first suggestion that crotalid phospholipases may achieve greater structural complexity in solution than the dimeric state. Protease activity, as evidenced by hydrolysis of six natural and synthetic substrates, was distributed throughout the first four fractions, suggesting the presence of multiple proteases with a wide range of molecular weights and with differing substrate specificities. Small myotoxins are absent from the venom of these populations. This is consistent with data from two Utah C. v. lutosus populations, but contrasts with all existing information on the venoms of conspecific taxa C. v. viridis, C. v. concolor, and C. v. helleri. Based on experience with other rattlesnake venoms, the fifth S-200 fraction probably consists of hypotensive peptides. No evidence was found for the presence of a crotoxin homolog in this venom.

It is difficult to explain the venom similarities between C. atrox and C. s. scutulatus (Type B) venom, and the profound, discrete differences between the two types of C. s. scutulatus venom. One possible explanation is that Type B venom snake populations have arisen in central Arizona through a series of recent hybridization events between C. atrox and C. s. scutulatus. While we are unaware of any published reports of wild-caught hybrids between these two species the absence of post-mating isolating mechanisms between the two species is clear, given

the evidence presented in our paper (12).

Because of currently accepted ancestral relationships among crotalids and reports of neurotoxicity in people bitten by L. m. muta, we were interested in examining its venom for crotoxin homologs. Using a combination of gel-filtration, SDS-PAGE, LD_{50} assays and immunological assays, we found no evidence for a crotoxin homolog in the venom we examined. We did observe a high molecular weight protein that when injected i. v. into mice it induced effects characteristic of gyroxin, from C. d. terrificus venom. However, the Lachesis protein appears to be nearly twice as large as gyroxin (≈60,000 vs 33,500) and is more toxic (LD_{50} = 0.07μg/g), but promotes the same bizarre, rapid barrel-rolling motions. The amino acid sequence of its amino terminus shows considerable similarity to the amino terminus of gyroxin and other serine proteases, some of which are responsible for processing peptide hormones and clotting protein precursors. It would be interesting to know the distribution of this protein in different snake venoms and how it induces the barrel-rolling motion.

An amino acid sequence comparison was carried out with the basic subunit of crotoxin and with 3447 sequences in the Protein Identification Resource at NBRF, which yielded a mean similarity score of 21.2, and 532 sequences with a score greater than 27. The five most similar sequences were phospholipases A_2 from crotalid and viperid venoms, with similarity scores ranging from 436 down to 388. Nineteen of the next 22 most closely related sequences were phospholipases A_2 from elapid venoms (similarity scores from 267 to 192).

The basic subunit of the crotoxin homolog from the venom of C. v. concolor displays 100% homology with the basic subunit of crotoxin over the N-terminal 43 residues. Percentage homologies with the acidic subunit of crotoxin and with other crotalid and viperid phospholipases are as follows: acidic subunit, 56.6% (estimated over 76 known residues of the acidic subunit); Bitis caudalis phospholipase, 52.5%; Crotalus adamanteus phospholipase, 50.0%; Crotalus atrox, 49.2%; Bitis nasicornis phospholipase, 49.2%; Bitis gabonica phospholipase, 47.5% and Trimeresurus okinavensis phospholipase, 47.2%. There is greater similarity between the acidic subunit of crotoxin and nontoxic, homodimeric phospholipases from C. adamanteus and C. atrox than between the two crotoxin subunits. The basic subunit of crotoxin and its homolog from the venom of C. v. concolor show greater similarity with the toxic monomeric phospholipase A_2 from B. caudalis than with any known crotalid phospholipases. This implies that toxicity evolved in phospholipases prior to the divergence of the Crotalidae and the Viperidae. Furthermore, the development of toxicity may have preceded the development of homo- and/or heterodimerism in phospholipases.

Sequence positions 24-32, near the amino terminus of the A-chain is at the interface between monomers in homodimeric phospholipases A_2 and may constitute part of the subunit binding site. Sequence positions 41-52, contain the phospholipase A_2 active site and Asp-48, which is specifically implicated in Ca^{2+} binding. Homology of the C-chain is maximal with phospholipase A_2 from the venom of the eastern diamondback rattlesnake (C. adamanteus)(Z=7.5).

All cysteine residues common to both toxic and nontoxic phospholipases appear to have been retained in the acidic subunit. It seems probable, therefore, that the disulfide bonds correspond with assignments made by Heinrikson for group II phospholipases (42).

The low pI of the acidic subunit (3.2 as opposed to 9.7 for the basic subunit), has been produced partly by the exclusion of three segments (positions 1-22, 62-70, and 105-111) containing a total of seven basic residues and only one to two acidic residues. These segments are believed to interact with phospholipid substrates in intact, nontoxic, crotalid phospholipases (J. Maraganore, personal communication). From the sequence comparison data it is not possible to determine the amino acid residues involved in the cleavages to generate the three chains. Clearly, it will be important to determine the sequence of the whole precursor in order to assign the sites of cleavage. It will be of interest to know how these cleavages relate to cleavages of precursors of other bioactive peptides.

Homology of both the acidic and basic subunits is greatest with nontoxic, crotalid phospholipases, which exist in solution as stable dimers with dissociation constants of 10^{-9}-10^{-11} M . Crotoxin exhibits maximal toxicity only as a dimer, despite the fact that it dissociates upon reaching the target tissue. That is, neurotoxicity is reduced about 10-fold if the dissociated basic subunit is injected alone. While retention of the subunit binding site by the acidic subunit further suggests its origin from a homodimeric phospholipase and suggests that the two active sites of the subunits face on another as proposed (43), the loss of enzymatic activity in the acidic subunit is intriguing. Randolph et al. (44) report that the dimeric phospholipase A_2 from C. atrox is completely destabilized when the amino-terminal 10 residues of the protein are removed with cyanogen bromide. In the acidic subunit, 22 residues are missing from the amino terminus.

The manuscript describing the amino acid sequence of the acidic subunit B-chain of crotoxin (17), completes our sequencing work on crotoxin. The sequence reported in the above paper is consistent with the final 24-residues reported earlier (2), except for position 20. Using automated Edman degradation we earlier found glycine at that position, whereas with mass spectrometry we found glutamic acid. Re-examination of the original Edman degradation results are consistent with the glycine identification. We can only attribute the difference to the presence of two different isoforms, one of which was sequenced by each of the methods. Bouchier et. al. (45) sequenced the cDNA encoding both subunits of crotoxin, including the B-chain and found the sequence identical to that reported here, with only glutamic acid at position 20. These workers did identify glutamine at position 68, which presumably cyclizes to pyroglutamate in the mature protein. The B-chain reported here shows 91% identity with the comparable segment from Mojave toxin; 77% and 74% identity with the comparable segment of non-toxic phospholipase A_2 from the venom of C. adamanteus and C. atrox, respectively; and only 49% identity with that portion of the basic subunit chain of crotoxin.

It was tempting to interpret the differences in CD and fluorescence spectra on intact crotoxin and the sum of subunit spectra as evidence for a large conformational change upon

complex formation between the acidic and basic subunits of all four proteins. Further studies showed however, that exposure of intact crotoxin to urea and subsequent removal by dialysis resulted in spectra (CD, FTIR, fluorescence) that were virtually identical to native intact crotoxin. This suggested that the changes seen in the spectra generated from reassociated, subunits, resulted from changes during subunit isolation and not merely from urea-induced conformational changes. What is suggested is that the mutual presence of the subunits is required for their proper folding upon removal of the denaturant, and reassembly into native oligomeric toxin. More definitive studies will require a subunit isolation procedure that does not alter subunit structure.

Fluorescence maxima of the tryptophans were not markedly shifted from subunits to intact toxins, even though there was a quenching of intrinsic fluorescence in intact material. This indicated that tryptophans remained in relatively polar environments in the dimeric toxins as well as in isolated subunits. While intact crotoxin, reconstituted from isolated subunits, exhibited obvious spectral differences from native crotoxin, they differed little in toxicity, suggesting that only limited regions of the protein are involved in these activities.

Toxicity values determined for the myotoxins fall around the range reported for E toxin (C. h. horridus) in the presence of acetate (Allen et al., 46). It may be argued that because of chromatography using acetate buffers, the basic amino acid side chains in these myotoxins have acetate bound as a counter-ion; hence their toxicity. Since the eluting cation exchange buffer contained only 20 mM acetate and 2 M NaCl, it is likely that Cl^-, rather than acetate, constituted the bulk of the counter-ion. Ownby (unpublished observations) has reported similar values for myotoxin a where no acetate was used in any purification step. We find the high LD_{50} value (low toxicity) reported (46), for E toxin in the absence of exogenous acetate (6.3 µg/g) difficult to explain. It may possibly be attributed to the one-step purification procedure they employed. Trace contamination with proteases might account for gradual loss of toxicity experienced previously with this toxin (Sullivan and Geren, 47).

The water soluble EDC appears to covalently cross-link the two subunits of crotoxin. Recovered product has the expected amino-terminal amino acids, amino acid composition, behavior on SDS-PAGE and a greatly reduced number of lysines. It has decreased immunological cross-reactivity toward both poly- and monoclonal antibodies raised to the basic subunit of crotoxin. This work is consistent with the results of Hendon and Tu (25), suggesting that crotoxin dissociation is required for expression of neurotoxicity. It does not however rule out the possibility that modification of essential residues result in the loss of toxicity. What is needed is a cleavable cross-linking reagent that effectively cross-links the two subunits of crotoxin.

A sensitive, reliable method for the continuous measurement of acetylcholine and choline in in vitro reactions is sorely needed. We still feel that luminometry is the best procedure for obtain the sensitivity needed, but it is currently fraught with experimental difficulties that

have yet to be resolved.

Results obtained in collaborative experiments with Lance Simpson's laboratory, which examined the interaction between crotoxin and the mammalian neuromuscular junction, suggested that crotoxin - but not the isolated subunit - binds avidly to the mammalian neuromuscular junction. The binding step is followed by a poisoning step, and there may be intermediate events as well. However, the data do not currently support the concept of a receptor-mediated endocytosis step that is interposed between binding and paralysis.

Venom fractionations were carried out using previously published procedures, for the most part. Our preparation of notexin from Notechis scutatus scutatus was the one major exception. We have spent considerable time in developing a fractionation procedure employing a combination of Sephacryl S-200HR followed by cation-exchange chromatography and another gel filtration step. Our final preparations of notexin and notechis II-5 appear homogeneous on SDS-PAGE and have i.v. LD_{50}-values of 0.005 and 0.04 µg/g, respectively. We have isolated what appears to be a new, toxic phospholipase in the process, which migrates just ahead of notexin on SDS-PAGE, cross-reacts with a MAb raised to pseudexin - as does notexin and notechis II-5 - and has an LD_{50} = 0.008µg/g. Characterization of this product is continuing.

CONCLUSIONS

1. Polyclonal antibodies raised to three different rattlesnake neurotoxins indicated that all were equally cross-reactive and all could neutralize the lethality of any of the toxins. These results suggest that crotalid neurotoxins can be detected and neutralized by polyclonal antibodies raised against any intact toxin or basic subunit in this group of homologous toxins.

2. All snake phospholipase A_2 neurotoxins fall into two main antigenic classes. One is composed of all the elapid toxins examined (textilotoxin, taipoxin, notexin, pseudexin and ß-bungarotoxin). Beta-bungarotoxin seems to be in a immunological subclass of its own. The second antigenic class is composed of crotalid and viperid phospholipase A_2 neurotoxins (crotoxin, concolor toxin, Mojave toxin, vegrandis toxin, ammodytoxin and caudoxin). The viperid toxins, caudoxin and ammodytoxin, appeared to be in an immunological subclass apart from the crotalid toxins. These data suggest that the snake phospholipase A_2 neurotoxins have certain common epitopes and , in some instances, common neutralizing epitopes.

3. The four monoclonal antibodies and rabbit polyclonals raised against the basic subunit of crotoxin have been further characterized for their cross-reactivity against each other, in the first step of attempting to determine their binding epitopes. One of the monoclonal antibodies is a potent neutralized of crotoxin's lethality and phospholipase activity. Another, is a non-neutralizer, but is able to inhibit the phospholipase activity by up to 90%. The availability of neutralizing and non-neutralizing MAbs provide another approach for determining the structural basis of crotoxin toxicity. Additional studies may provide insight into the antigenic

regions of crotoxin and define non-toxic peptides useful for pre-bite immunization studies.

4. A crotoxin homolog is present in the venom of C. v. concolor, C. s. scutulatus (Type A venom), C. vegrandis, and C. d. collilineatus, but none was found in venom from either C. v. lutosis, Lachesis muta muta, or C. atrox/C. atrox-C. s. scutulatus (Type B venom) hybrids. With Lachesis we did demonstrate the presence of a toxic, 60 kd gyroxin-analog, which produced barrel-rolling motions in mice.

5. Determination of the primary sequences of the acidic and basic subunits of crotoxin have been completed. While the acidic subunit lacks phospholipase activity, it was clearly derived from an ancestral phospholipase gene.

6. Early structural studies on crotoxin and related crotalid dimeric, presynaptic neurotoxins indicated that major conformational changes occurred in individual subunits upon formation of the dimeric toxins. Additional studies showed however, that when intact crotoxin was exposed to urea and subsequently re-isolated, their spectra were indistinguishable from unexposed, intact crotoxin. Findings which suggest that the spectral changes observed in isolated subunits were artifacts, resulting from structural changes that occurred during subunit isolation by urea ion-exchange that was not easily reversed under our in vitro conditions.

7. Crotoxin cross-linked by a different cross-linker than used by Hendon and Tu, also proved to be non-toxic. These results do not rule out the possibility that modification of essential residues result in the loss of the observed toxicity. What is needed is a cleavable cross-linking reagent that effectively cross-links the two subunits of crotoxin.

8. After unsuccessfully identifying any crotoxin basic subunit clones from our cDNA library, and acidic subunit clones with extensive sequence differences, we concluded that the preparation of new libraries were in order. Both cDNA and genomic libraries from C. s. scutulatus are in preparation.

9. Luminesce methodology still appears to be the most desirable method for choline and acetylcholine detection if the experimental problems can be resolved.

10. Collaborative efforts with Lance Simpson have provided us with another technique with which to examine the interaction of presynaptic neurotoxins with mammalian neuromuscular junctions. This methodology indicates that intact crotoxin, but not the individual subunits, bind with great affinity to the mammalian neuromuscular junction.

11. In our purification of toxins, we developed an updated purification procedure for notexin and notechis II-5 from N. s. scutatus. In addition, we appear to have isolated a new, toxic phospholipase with an LD50 = 0.008µg/g in mice.

RECOMMENDATIONS

1. Continue the characterization of the monoclonal antibodies and attempt to identify the

epitope recognized by the neutralizing monoclonal antibody. Also attempt to identify antigenic regions recognized by the polyclonal antibodies.

2. Continue our collaboration with Dr. Keith Ward on the determination of the crystal structure of Mojave toxin that is in progress. Attempt to co-crystalize the F_{ab}-fragment of the crotoxin neutralizing MAb and Mojave toxin. Expand this collaboration when additional purified toxins and subunits become available.

3. Continue attempts to establish an in vitro system using synaptosomes and bioluminescence for examining the biological effects of presynaptic neurotoxins on acetylcholine release. Maintain the collaboration with Dr. Lance Simpson, whose phrenic nerve-hemidiaphragm system may help elucidate the mechanism of action of the phospholipase A_2 neurotoxins.

4. Continue to characterize the cDNA and genomic clones of C.s. scutulatus, and attempt to get them expressed for future site-specific mutagenesis studies.

5. Further characterize the new toxic phospholipase A_2 found in the venom of N. scutatus during the purification of notexin and notechis II-5.

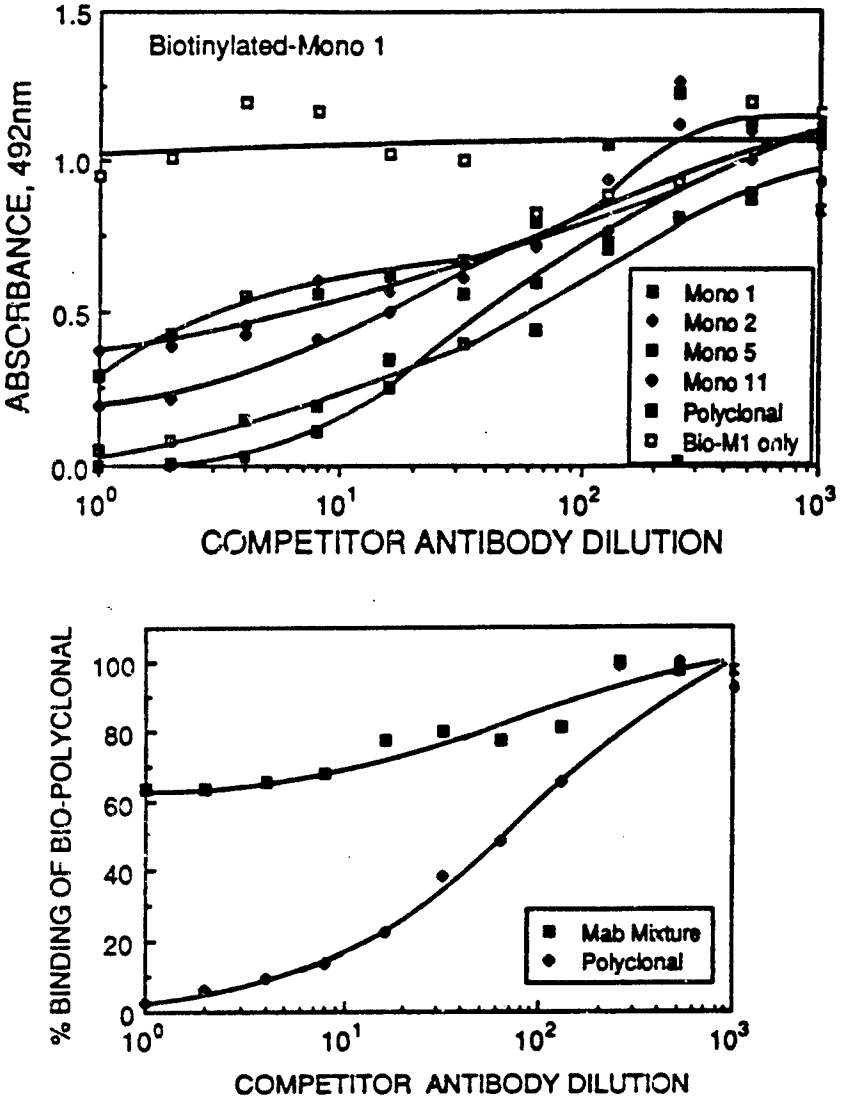


Fig. 1. Competition of MAbs and polyclonal Abs for the binding of biotinylated-MAb 1 to the basic subunit of crotoxin. Microtiter wells coated with the basic subunit of crotoxin were incubated with decreasing concentrations of competitor Abs in the presence of biotinylated-MAb 1. Bound biotinylated-MAb was detected by an avidin-biotin-peroxidase complex as described (Kaiser and Middlebrook, 7). Concentration of biotinylated-MAb was 50 ng/well and initial unlabeled Ab concentration was 3 µg/well.

Fig. 2. Competition of a mixture of four MAbs (raised against the basic subunit of crotoxin) for the binding of biotinylated-polyclonal Abs (raised in a rabbit against the basic subunit of crotoxin). Well coating, dilutions, and detection were as described in legend for Fig. 1. Concentration of biotinylated-polyclonal antibodies was 25 ng/well and the initial concentrations of polyclonal antibodies and the MAb mixture were 3 and 12 µg/well, respectively.

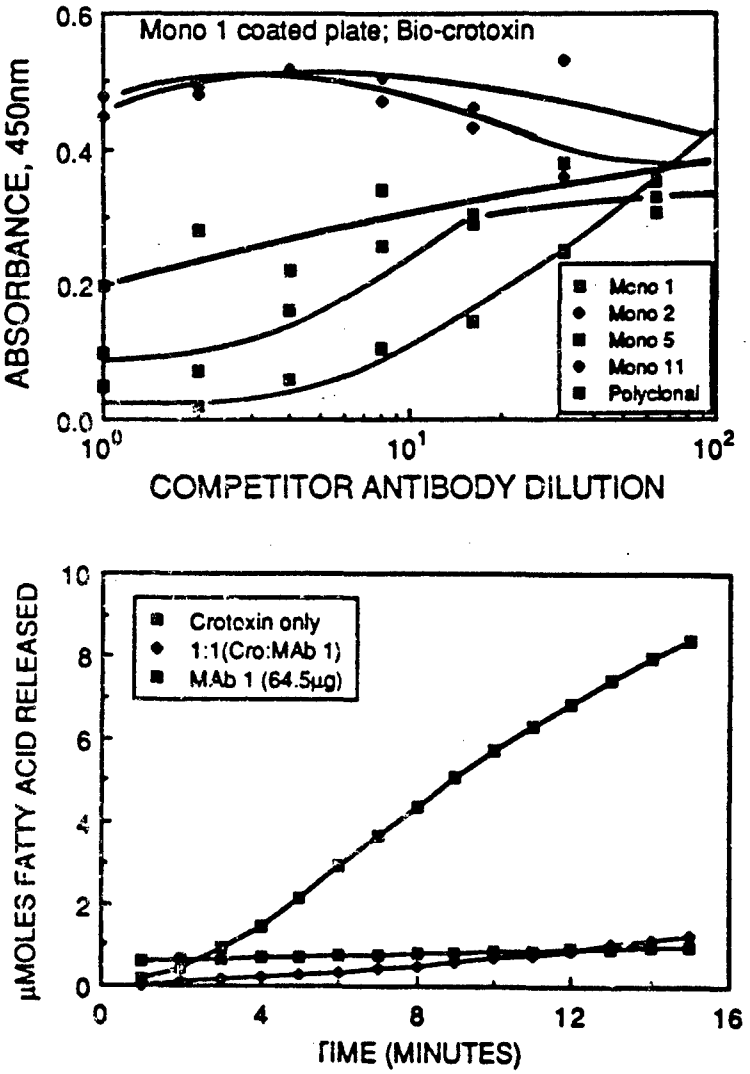
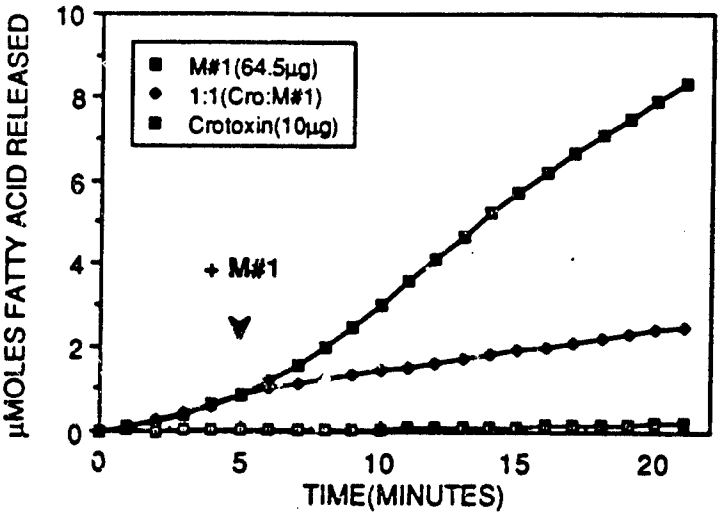


Fig. 3. Competition of MAbs and polyclonal Abs for the binding of biotinylated-crotoxin to wells coated with MAb 1. Biotinylated-crotoxin is mixed with different dilutions of the test Ab and incubated overnight. The mixtures are then added to wells coated with MAb 1. Lack of competition between the test Ab and MAb 1 for the same epitope should not affect biotinylated-crotoxin binding. Bound biotinylated-crotoxin was detected as in Fig. 1. Biotinylated crotoxin was 50 ng/well and initial concentrations of mono- and polyclonal antibodies were 1 and 8 µg/well, respectively.

Fig. 4. Phospholipase activity of crotoxin on phosphatidylcholine at a 2:1 molar ratio of Triton X-100-phospholipid in the presence and absence of MAb 1. Fatty acids released were titrated and maintained at pH 8 with dilute NaOH under nitrogen at 37°, using a radiometer titration apparatus (Aird and Kaiser, 5). One ml of substrate was used per reaction. Phospholipase activity was assayed after the addition of 10µg of crotoxin and any MAbs at the indicated concentration. When Abs were used, they were preincubated with the phospholipase for 15 min at 37° before being assayed unless indicated.



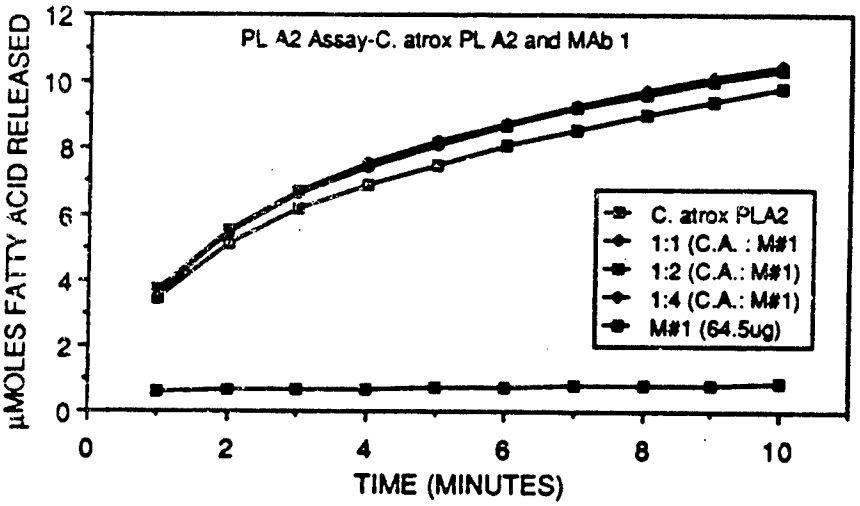


Fig. 5. Phospholipase activity of crotoxin in the absence and presence of MAb 1 added 5 min into the reaction. Other conditions as described as in Fig. 4.

Fig. 6. Phospholipase activity of phospholipase A_2 from C. atrox on phosphatidyl choline in the absence and presence of different concentrations of MAb 1. Other conditions as described in Fig. 4.

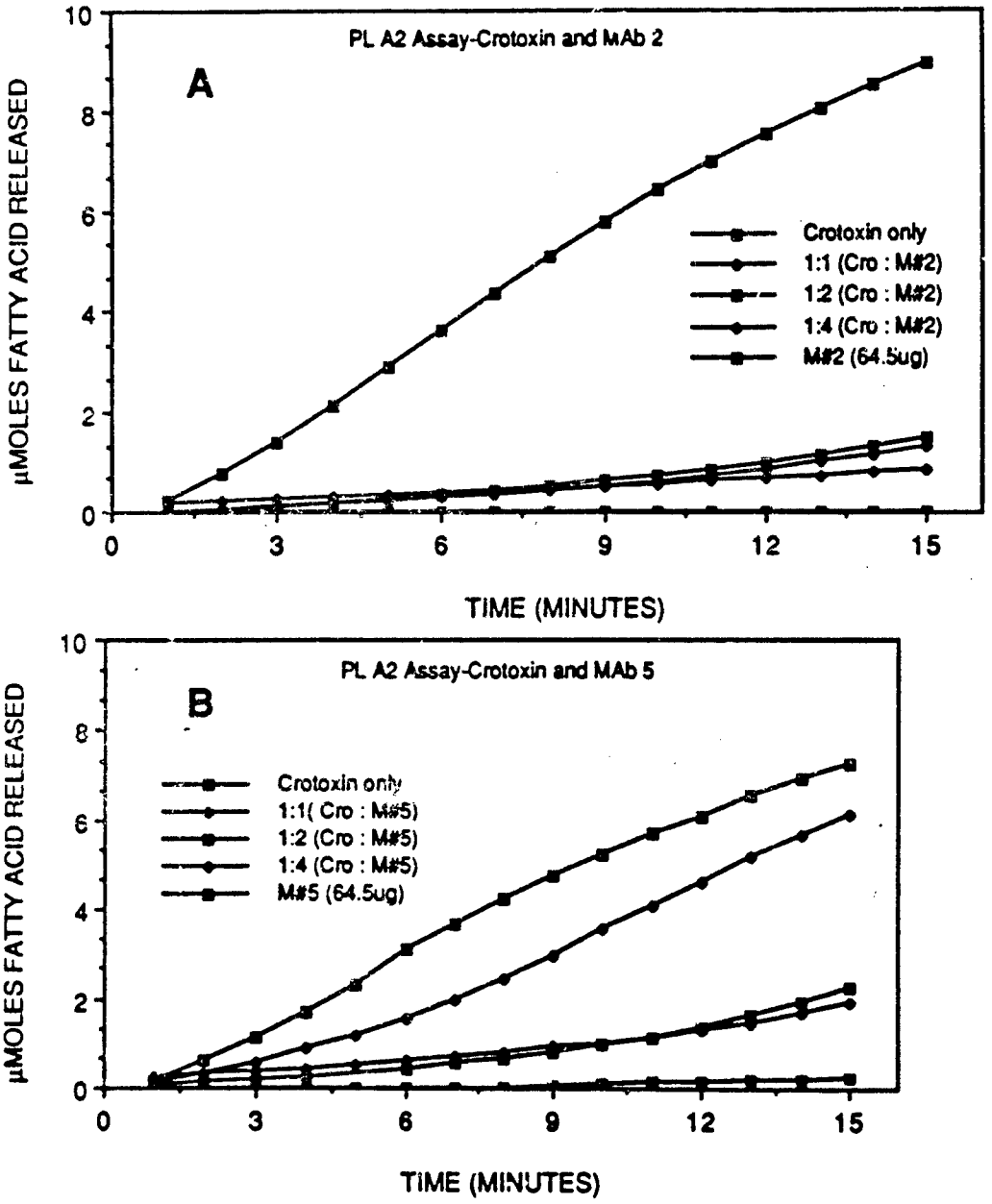
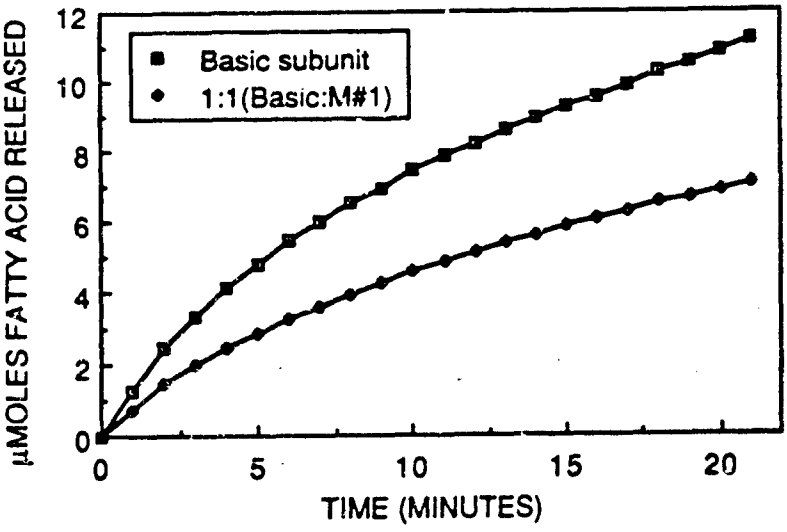


Fig. 7. Phospholipase activity of crotoxin in the absence and presence of different concentration of (A) MAb 2 and (B) MAb 5. Other conditions as described in Fig. 4.



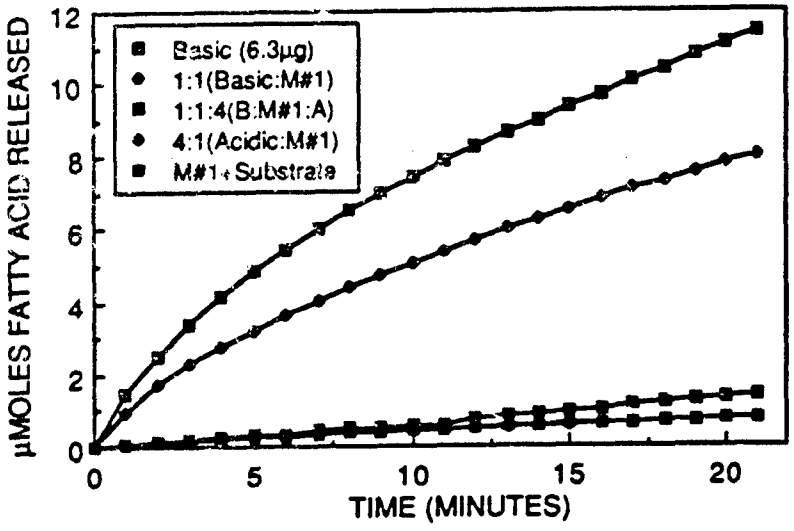


Fig. 8. Phospholipase activity of the basic subunit of crotoxin (6.3µg) on phosphatidyl choline in the absence and presence of an equal molar amount of MAb 1. Other conditions as described in Fig. 4.

Fig. 9. Phospholipase activity of the basic subunit of crotoxin (6.3µg) in the absence and presence of equal molar amounts of MAb 1; and in the presence of both MAb 1 and acidic subunit of crotoxin. Other conditions as described in Fig. 4.

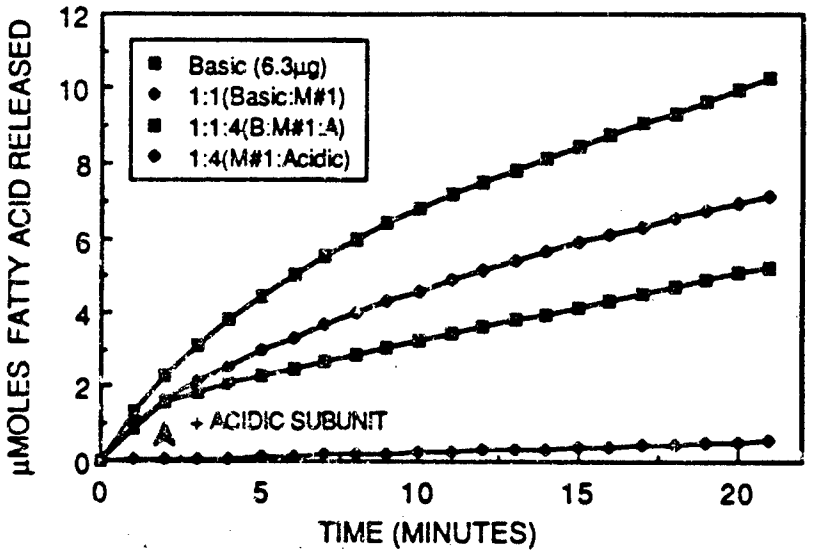


Fig. 10. Phospholipase activity of the basic subunit of crotoxin (6.3µg) in the absence and presence of equal molar amounts of MAb 1. In one reaction a four-fold excess of the acidic subunit was added to a basic subunit-MAb 1 mixture. Other conditions as described in Fig. 4.

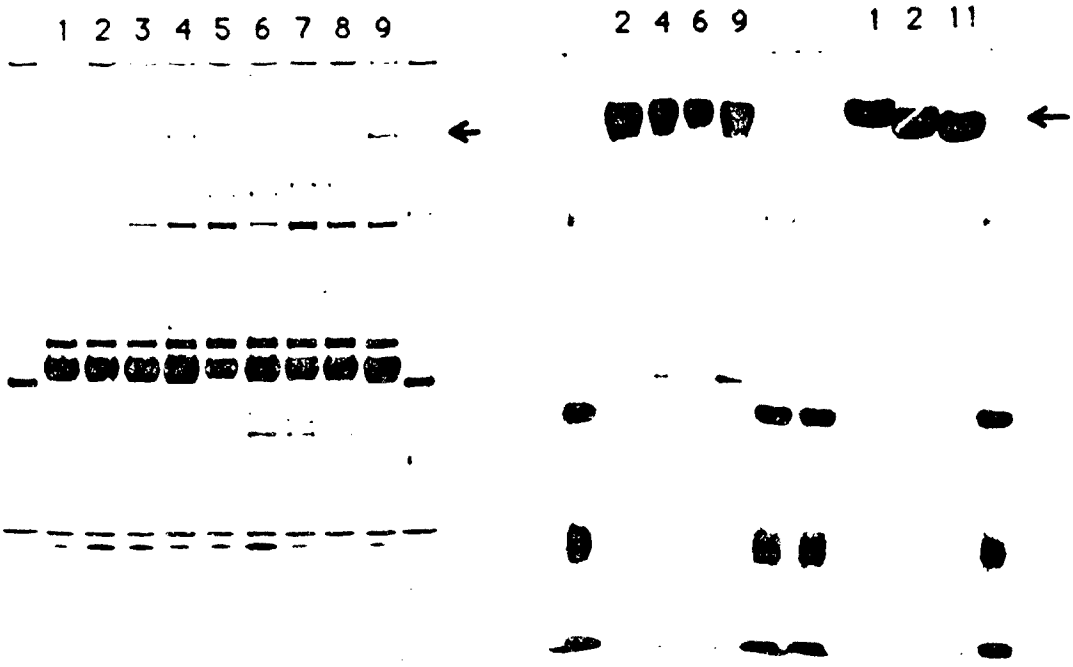


Fig. 11. Non-reducing SDS-PAGE (7.5% acrylamide) of anti-idiotype ascites fluid from mice inoculated i.p. with hybridoma cells generated against the purified, intact crotoxin neutralizing monoclonal antibody prepared by Kaiser and Middlebrook (8). About 20µg of protein was run per slot. The antibody region of the gel is indicated by the arrow. Assigned number and reference number are as follows: 1, 4D10-1G6-1D2 (5-12-87); 2, 7E11-2D4-2C8 (5-12-87); 3, 6G9-2E4-1D4 (5-12-87); 4, 10E6-1G2-1B5 (5-12-87); 5, 4F9-2B4-2D5 (5-20-87); 6, 7B5-2E11-1B6 (5-12-87); 7, 10D8-1E7-1B8 (3-11-87); 8, 7E11-2D4-1B9 (1-9-87); 9, 7F9-1F4-1C2 (3-4-87).

Fig. 12. Non-reducing SDS-PAGE (7.5% acrylamide) of four purified, anti-idiotype monoclonal antibodies. Monoclonal antibodies from samples 2, 4, 6, and 9 were recovered from crude ascites fluid by protein A chromatography. The antibody region of the gel is indicated by the arrow. Purified monoclonal antibodies 1, 2, and 11, raised against the basic subunit of crotoxin are shown for comparison (ref. 8). About 20µg of protein was run per slot.

TABLE A. BIOTINYLATED-MONOCLONAL ANTIBODY

BIO-Ab	1	2	5	11	P
1	++++	++	++	+++	+++++
2	+	++++	+	+++	+++++
5	+++	+	++++	+	+++++
11	+	++++	+	++++	+++++

COMPETITOR ANTIBODY

TABLE B. BIOTINYLATED-CROTOXIN

Ab COATING PLATE	1	2	5	11	P
1	++++	0	++	0	+++
2	0	++	+	++++	+++
5					
11	0	++++	++	+++++	+++

COMPETITOR ANTIBODY

Table 1. Summary and semiquantitation of the data showing the competition of (A) different antibodies with biotinylated-MAb for immobilized basic subunit of crotoxin; and (B) biotinylated-crotoxin for immobilized MAbs.

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